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<b>(21) International Application Number:</b> PCT/US92/02724 <b>(22) International Filing Date:</b> 3 April 1992 (03.04.92)  <b>(30) Priority data:</b> 680,330 4 April 1991 (04.04.91) US  <b>(71) Applicant:</b> IBEX TECHNOLOGIES, INC. [CA/CA]; 6100 Avenue Royalmount, Montreal, Quebec H4P 2R2 (CA).  <b>(71)(72) Applicant and Inventor:</b> ZIMMERMANN, Joseph, J. [US/US]; 13450 Nicolet Avenue, Elm Grove, WI 53122 (US).  <b>(72) Inventors:</b> LEWIS, N., Tracey ; 3820 Brahms, Brossard, Quebec J4Z 2X1 (CA). HEFT, Robert, A. ; 2320 Ward Avenue, Ville St. Laurent, Quebec H4M 2V5 (CA).		<b>(74) Agents:</b> PABST, Patrea, L. et al.; Kilpatrick & Cody, 100 Peachtree Street, Suite 3100, Atlanta, GA 30303 (US).  <b>(81) Designated States:</b> AT (European patent), AU, BE (Euro- pean patent), CA, CH (European patent), DE (Euro- pean patent), DK (European patent), ES (European pa- tent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (Euro- pean patent), MC (European patent), NL (European pa- tent), SE (European patent).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title:</b> ENZYMATIC NEUTRALIZATION OF HEPARIN  <b>(57) Abstract</b>  A heparinase formulation derived from <i>F. heparinum</i> which meets all requirements for a clinical reagent that can eliminate heparin interference of normal blood function has been developed. The heparinase, derived from <i>F. heparinum</i> , is free of a component that inhibits coagulation. It is stable under normal manufacturing, shipping and clinical storage conditions for a least one year. The heparinase is useful <i>in vitro</i> to eliminate the interference in hematological assays due to the presence of heparin. The heparinase is also useful for the <i>in vivo</i> neutralization of heparin during surgical procedures. Advantages of this preparation are that it achieves neutralization faster and more completely than previously available compositions and is stable for long periods of time.		

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## ENZYMATIC NEUTRALIZATION OF HEPARIN

### Background of the Invention

This invention relates to a method for treating mammalian blood and plasma with a commercially viable bacterial heparinase preparation to eliminate the interference of normal blood function resulting from the presence of heparin.

Heparin is a sulfated glycosaminoglycan with a backbone comprised of alternating hexuronic, either L-iduronic or D-glucuronic and D-glycocyamine residues joined in alternating 1,4 linkages. The heterologous nature of heparin is due to the varying degree and location of sulfate substitution on these residues giving rise to at least ten different monosaccharide building blocks within the polymer, as reported by Lindahl, et al., Biosynthesis of heparin TIBS 11(5):221-225 (1986). The heterogeneity and high degree of sulfate substitution, greater than 2.6 sulfates per disaccharide unit, gives heparin a high protein binding capacity resulting in the inhibition or activation of several enzyme systems (Sakamoto and Sakamoto, "Heparin and bone metabolism: Effects of heparin on bone collagenase release and activity and an application of heparin-sepharose affinity chromatography for *in vitro* study of bone resorption" in Chemistry and Biology of Heparin (Elsevier/North Holland Press, Amsterdam 1981)). Protein-heparin associations are most frequently due to electrostatic interactions although associations due to tertiary and secondary structure interactions corresponding with specific oligosaccharide sequence regions have also been observed. The most thoroughly studied sequence specific interaction is the stabilization of the antithrombin 3 (AT III) - thrombin complex which results in the inhibition of coagulation, as discussed by Rosenberg and Damus, J. Biol. Chem. 248:6490-6505 (1973).

Heparin is widely used as an anticoagulant in conjunction with invasive surgical procedures and dialysis procedures, to prevent clotting in

intravenous lines and in the treatment of thrombolytic disorders. 5-10% of all hospitalized patients have heparin in their blood. Recently, low molecular weight heparins, chemical derivatives of native heparin, have been investigated as potential anticoagulants where the primary mechanism of action is the inhibition of factor Xa, as reported by Choay, et al., Thrombosis Res. 11:240 (1980).

Patients receiving heparin therapy or who have been exposed to heparin through intravenous lines are frequently tested by a variety of means for the assessment of their hematological status or to monitor heparin therapy, itself. For example, activated clotting time assays are performed at 20 minute intervals during extracorporeal procedures to ensure adequate heparinization and the prevention of contact activated clotting. Heparin interferes with several routine hematological analyses. The presence of heparin in the bloodstream will prevent the identification of coagulopathies by standard coagulation assays which rely on clotting as the endpoint. These include activated partial thromboplastin time, prothrombin time, factor complement assays and activated clotting time. Heparin's electrostatic interactions with essential components of other tests, such as the polylysine substrate in the fibrinolysis assay, causes similar interference problems.

Several approaches have been attempted to circumvent the heparin interference problem. Ion exchange resins have been used to adsorb heparin from the sample prior to testing, as described by Cumming, et al., Thrombosis Res. 41:43-56 (1986). This method is non-specific, removing coagulation factors and other blood proteins in addition to heparin, thereby influencing the test result. The technique is also time consuming and cannot be readily used as a STAT test during surgery where rapid data acquisition is necessary.

Protamine sulfate has been used to neutralize heparin by electrostatic interaction and precipitation, as also reported by Cumming, et al., (1986). The protamine-heparin reaction is stoichiometric and requires an accurate titration to prevent adverse effects resulting from incomplete neutralization or from protamine sulfate's own anticoagulant properties. This method is cumbersome, prone to error and requires large sample volumes for accurate titration measurements. Protamine sulfate is not able to neutralize the anticoagulant effect of low molecular weight heparins.

Another approach is to supplement inhibited thrombin with additional thrombin or a substitute enzyme, such as reptilase, capable of catalyzing a similar reaction, as described by Funk, et al., Brit. J. Haematol. 21:43-52 (1971). This method may be successful for assays intended to monitor events subsequent to fibrin formation but is unsuitable for detecting coagulopathies in the coagulation pathway prior to the thrombin catalyzed reaction. Furthermore, heparin's effects on other components such as platelets, cannot be circumvented by these replacement enzymes.

The most desirable resolution to the heparin interference problem would be a method that could expeditiously and specifically remove heparin from blood samples immediately before the onset of the test. The additive used to accomplish this must function over a broad range of conditions. Heparin should be neutralized, nearly instantaneously, while the reagent, itself, should not impart any effects on blood components over a lengthy exposure period. A reagent demonstrating these characteristics could be used in conjunction with ACT assays which are performed immediately after sample procurement and on samples examined in the hematology laboratory which are liable to sit on the bench for up to one hour before processing. The reagent must function over a broad temperature range, 2 to 37°C, to accommodate samples including: those

stored on ice in the hematology laboratory, samples taken from patients undergoing cardiovascular surgery whose blood temperature is maintained at 30°C and samples from patients undergoing procedures such as dialysis which are carried out at normal body temperatures, 37°C. The heparin neutralizing reagent should also be concentration independent such that a single dose of the reagent will effectively neutralize a broad range of heparin concentrations exceeding clinically used quantities (up to 0.3 IU/ml for thrombolytic therapy, up to 1.5 IU/ml for dialysis therapy, and up to 6 IU/ml for cardiovascular surgery). Furthermore, the treated samples containing heparin should give a result identical to untreated samples that have not been exposed to heparin. A candidate reagent would be a degradative glucanase enzyme specific for heparin which had no other effect on the sample as measured by the intended test.

Hutt and Kingdon, J. Lab. Clin. Med. 79:1027 (1972), attempted to use a heparinase from *Flavobacterium heparinum* to treat plasma samples prior to performing PTT analysis. The authors noted the requirement for purifying the heparinase from crude extracts of *F. heparinum* to reduce an interference effect from the bacterial source. However, their data indicated their inability to completely remove the interfering moiety. A protein preparation containing 0.008 IU heparinase was insufficient in neutralizing 0.1 IU/ml heparin while a preparation containing 0.04 IU heparinase caused an extended PTT time. The success of this preparation relies on an accurate titration of the enzyme and would be limited in the amount of heparin that could be neutralized.

U.S. Patent No. 4,795,703 to Folkman, et al, describes using heparinase from *F. heparinum* to develop a method for making quantitative heparin determinations in whole human blood samples using an activated clotting time assay. They were similarly unable to completely remove an interfering moiety from their enzyme preparations. Heparinase treated

samples originally containing heparin demonstrated ACT times 16% longer than untreated samples that had not been exposed to heparin. This effect was not detrimental to their process as quantitative heparin determinations relied on comparing test results to a standard curve which presumably took into account the influence of the enzyme preparation on the test result.

Neither group identified the *F. heparinum* moiety responsible for influencing the test results nor demonstrated its resolution from the active *F. heparinum* component responsible for heparin neutralization. The authors of these papers could not differentiate whether their preparations contained an additional molecule from *F. heparinum* that acted as an anticoagulant or whether heparinase, itself, has anticoagulant properties when used in excess or in combination with heparin. The recent characterization of myxalin, a glycoprotein from a Gram negative bacterial source, demonstrates that it possesses anticoagulant properties associated with its carbohydrate moiety, as reported by Akoum, et al., Thrombosis Res. 60:9-18 (1990). Evidence strongly indicates that heparinase is also a glycoprotein, thereby substantiating its potential to have similar characteristics.

Bohmer, et al., Thrombosis Res. 60: 331-335 (1990) have used heparinase III from an undetermined, unavailable bacterial source to neutralize heparin prior to aPTT and PT assays and examined its potential to neutralize heparin's inhibition of thrombin mediated fibrinogen degradation. Incubations of the enzyme with citrated plasma at 37°C for five minutes and with whole blood at 37°C for 15 minutes were used to achieve the desired heparin neutralization. The optimal activity profile of heparinase III, pH 7.6, [NaCl] = 0.03 and T = 45°C (16), may have necessitated the inclusion of an incubation period in the reported protocol.

Neither of these three groups presented evidence for the stability of their heparinase formulation, an essential component of a clinical product.

One of heparin's primary therapeutic uses is to prevent coagulation during cardiovascular surgery while the patient's blood is circulating through an extracorporeal circuit. Approximately ten times the normal thrombolytic dose of heparin is used in this application, requiring post-operative neutralization by the administration of protamine. Since protamine reversal is associated with several complications and requires titration, an alternative approach to the heparin/protamine control of hemostasis is desirable. One method may be to replace protamine reversal with heparinase, directly injected into the patient after bypass.

Langer and co-workers investigated the *in vivo* effects of heparinase in animal models, as reported by Klein, et al., J. Lab. Clin. Med. 102:828-837 (1982) and Langer, et al., Trans. Am. Soc. Artif. Intern. Organs 28:387-390 (1982). Heparinase with a specific activity of 0.58 IU/mg was injected into rabbits that had received heparin and aPTT assays were used to measure coagulation over time. An accelerated heparin clearance was noted as compared to rabbits which did not receive heparinase. However, coagulation times were still three times baseline, 15 minutes after the heparinase injection and returned to baseline only 1 h later, the same period of time for the control animal which did not receive a heparinase injection. Langer was able to achieve better reversal by pumping blood through an immobilized heparinase reactor. Following this result, the Langer group abandoned the idea of direct heparinase injection and focused on developing the extracorporeal use of heparinase to replace protamine.

It is therefore an object of the present invention to provide a heparinase preparation which can be used to quickly and completely

neutralize heparin in a wide range of concentrations, both *in vitro* and *in vivo*.

It is a further object of the present invention to provide a heparinase preparation which is free of any contaminants altering coagulation.

It is a further object of the present invention to provide a heparinase preparation which is stable for an extended period of time at room temperature.

### Summary of the Invention

A heparinase formulation derived from *F. heparinum* which meets all requirements for a clinical reagent that can eliminate heparin interference of normal blood function has been developed. The heparinase, derived from *F. heparinum*, is free of a component that inhibits coagulation. It is stable under normal manufacturing, shipping and clinical storage conditions for at least one year. The heparinase has an optimal activity profile close to physiological conditions: pH = 6.5 to 7.0; [NaCl] = 0.1, and T = 37°C. The preparation achieves the intended neutralization over a broad range of conditions encompassing all of those likely to be encountered in a clinical setting.

The heparinase is useful *in vitro* to eliminate the interference in hematological assays due to the presence of heparin. The heparinase is also useful for the *in vivo* neutralization of heparin during surgical procedures. Advantages of this preparation are that it achieves neutralization faster and more completely than previously available compositions and is stable for long periods of time.

### Brief Description of the Drawings

Figure 1 is a graph of the activated clotting time (seconds) versus time after heparinase injection (minutes) for a control rabbit receiving 250 IU heparin/kg but not heparinase (open circles) and a rabbit receiving 250 IU heparin/kg followed five minutes later with 2.5 IU heparinase/kg (closed circles). The baseline ACT is indicated as (....) The ACT of the treated rabbits was monitored over ninety minutes.

### Detailed Description of the Invention

A heparinase formulation derived from *F. heparinum* which meets all requirements for a clinical reagent that can eliminate heparin interference of normal blood function has been developed. Heparinase itself has been determined not to be an anticoagulant. In pursuing this aim, the heparinase from *F. heparinum* was completely separated from a component that inhibits coagulation. A method to formulate heparinase so that it is stable under normal manufacturing, shipping and clinical storage conditions for at least one year has also been developed. Heparinase from *F. heparinum* has an optimal activity profile which is closer to physiological conditions, rendering this enzyme suitable for the intended applications: pH = 6.5 to 7.0; [NaCl] = 0.1, and T = 37°C. The preparation described herein achieves the intended neutralization over a broad range of conditions encompassing all of those likely to be encountered in a clinical setting. A comparison of the results shown herein with published information demonstrates that this purified, stabilized heparinase provides the first commercially based product capable of resolving the heparin interference problem.

There are three operational components: (1) the preparation of anticoagulant free heparinase, (2) the stabilization and formulation of anticoagulant free heparinase, and (3) the use of anticoagulant free

heparinase to eliminate the interference in hematological assays due to the presence of heparin. The heparinase is also useful for the *in vivo* neutralization of heparin during surgical procedures.

*Preparation of anticoagulant free heparinase.*

Heparinase is isolated from cultures of the Gram negative bacterium *Flavobacterium heparinum*. *F. heparinum* is grown in a suitable nutrient medium containing heparin to induce heparinase synthesis according to the method of Galliher, et al., Appl. Environ. Microbiol. 41(2):360-365 (1981) or a similar method. The *F. heparinum* cells are concentrated and the heparinase released into solution by sonication, homogenization or an osmotic shock technique. Heparinase is then purified from this solution by one or more of the standard purification methods including: protamine sulfate precipitation, ammonium sulfate precipitation, hydroxylapatite chromatography, anion exchange chromatography or cation exchange chromatography, to produce a preparation with a specific activity of between 10 and 35 IU heparinase/mg protein. This material contains a moiety that inhibits coagulation, as measured by the standard aPTT and ACT assays.

The material is further purified by an affinity chromatography step using cellulose sulfate (Amicon™) or an equivalent polysulfated resin. The material is exposed to the resin under conditions in which the heparinase is adsorbed while the anticoagulant moiety remains in solution, for example, pH 7.0, conductivity between 3 to 12 mmhos. After washing the resin to thoroughly remove traces of the anticoagulant moiety, the heparinase is recovered by washing in a buffer of increased conductivity, for example pH 7.0, conductivity greater than 16 mmhos. Functionally equivalent solutions can be substituted for the pH 7.0, 3 to 12 and greater than 16 mmho solutions. The heparinase fraction recovered is free of the contaminating anticoagulant moiety.

*Heparinase clinical formulation.*

Heparinase, stored in buffered salt solutions normally used to carry out the heparinase reaction, is reported by Langer, et al., Science 217:261-263 (1982), to have a half life of 1 h at 37°C, 30 h at 23°C and 125 h at 4°C. As described below, several compounds have been examined for their ability to stabilize a soluble heparinase preparation. Of these, ammonium sulfate performed best, extending the enzymatic half life of heparinase 60 fold. High concentrations of sodium acetate and sodium sulfate also stabilized heparinase activity, but not to as great an extent as ammonium sulfate. The stabilizing agents, preferably the ammonium sulfate or other similar salt, are added to the heparinase in a ratio of 0.5 - 1.0 mg ammonium sulfate/IU anticoagulant free heparinase. Most clinical samples will contain in the range of 0.05 to 3.0 IU anticoagulant free heparinase.

*Elimination of heparin interference.*

An aliquot of freshly drawn whole blood or plasma recovered from blood is transferred into a tube containing the lyophilized heparinase preparation. The enzyme is allowed to dissolve into the sample and incubate for up to 60 minutes at 4-37°C. In general, heparinase neutralization is complete within the time required for the enzyme to dissolve, less than 15 seconds. For example, 0.05 IU heparinase completely neutralizes the heparin in a 0.4 ml whole blood sample containing 4.8 IU heparin in 15 seconds.

After incubation the sample is ready for processing by the intended hematological assay. The assay may be processed using standard protocols. The result obtained will be equivalent to a result obtained for a sample taken from the same patient that had not been exposed to either heparin or heparinase.

Alternately, heparinase at a dosage of 0.01 IU heparinase/IU heparin can be intravenously injected into a mammalian body. Within fifteen minutes of the heparinase injection, the effects of the heparin on hemostasis will be neutralized.

The present invention will be further understood by reference to the following non-limiting examples.

**Example 1: Heparinase preparation.**

100 L. *F. heparinum* was grown in a Chemap fermenter using a variation of the minimal media described by Galliher, et al., Appl. Environ. Microbiol. 41(2):360-365 (1981), the teachings of which are incorporated herein. The temperature was maintained at 23°C, and pH controlled to pH 7.0 by the appropriate addition of ammonium hydroxide. After 24 h incubation a volumetric heparinase activity of 2.5 IU/ml was achieved. The cells were concentrated by centrifugation and the heparinase released from the periplasmic space by a variation of the osmotic shock procedure described by Zimmermann, et al., U.S. Serial No. 07/203,235, the teachings of which are incorporated herein. The osmolate was processed by cation exchange chromatography as described by Zimmermann, et al. The resulting heparinase fraction was loaded directly onto a cellulose sulfate column (5.0 cm i.d. x 30 cm) and eluted with successive 1 L washes of 10 mM sodium phosphate, pH 7.0, 10 mM sodium phosphate, 200 mM NaCl, pH 7.0, 10 mM sodium phosphate, 400 mM NaCl, pH 7.0 and 10 mM sodium phosphate, 1000 mM NaCl, pH 7.0.

The results of the affinity chromatography step are shown in Table 1. Heparinase activity is recovered in the fraction eluting at 0.4 M NaCl while the anticoagulant moiety is in the unbound fraction. The heparinase recovered from this step is suitable for the intended use to eliminate heparin's effects on blood.

**Table 1: Purification of heparinase by affinity chromatography using cellulose sulfate<sup>TM</sup> (Amicon)**

sample	activity (IU)	specific activity (IU/mg)	anticoagulant activity* (aPTT ratio)
feed	1670	28.3	2.0
unbound fraction	83.3	ND	3.4
0.2 M NaCl fraction	0	ND	1.0
0.4 M NaCl fraction	1470	41.7	1.0
1.0 M NaCl fraction	0	ND	1.0

\* anticoagulant activity is measured by incubating a known quantity of material with normal human plasma for 15 minutes at 4°C and performing an aPTT test. The activity is expressed as a ratio of the aPTT of the plasma incubated with the material to the normal aPTT value for that sample (typically 24-28 seconds).

A further purification of the heparinase was achieved by hydroxylapatite column chromatography. The heparinase fraction collected from the cellulose sulfate chromatography step was dialyzed two times against ten volumes of 10 mM sodium phosphate, pH 7.0 and loaded onto a Biogel HTP<sup>TM</sup> (BioRad) column (1.6 cm i.d. x 10 cm). The protein was eluted with a linear gradient of 10 to 250 mM sodium phosphate and 0 to 0.5 M sodium chloride over twenty column volumes. Heparinase was recovered in a single protein peak eluting at 16 mM sodium phosphate and 0.19 M sodium chloride. SDS-PAGE analysis of this material indicates that it approaches homogeneity (greater than 98%). A summary of the heparinase recovery steps showing purification data and the removal of the anticoagulant moiety is shown in Table 2. Homogeneous heparinase recovered from the hydroxylapatite step described above was similarly able to neutralize heparin in human blood and plasma indicating that no other protein contained in the cellulose sulfate preparation, beside heparinase, is required for this use.

**Table 2: Purification of heparinase from *Flavobacterium heparinum* fermentations.**

sample	activity (IU) x 10 <sup>3</sup>	specific activity (IU/mg)	anticoagulant activity* (aPTT ratio)
fermentation	83.30	0.58	1.5
osmotic shock	65.00	3.33	1.75
cation exchange LC	29.17	28.3	2.0
affinity LC	14.67	41.7	1.0
hydroxylapatite LC	9.83	75.0	1.0

\* anticoagulant activity is measured by incubating a known quantity of material with normal human plasma for 15 minutes at 4°C and performing an aPTT test. The activity is expressed as a ratio of the aPTT of the plasma incubated with the material to the normal aPTT value for that sample (typically 24-28 seconds).

**Example 2: Stabilization of heparinase preparation.**

The heparinase preparation is stable under normal manufacturing, shipping and clinical storage conditions for at least one year. Stability is in part a function of additives, most preferably ammonium sulfate. Several compounds have been examined for their ability to stabilize a soluble heparinase preparation. The results are shown in Table 3.

**Table 3: Effect of additives on the stability of heparinase in solution at 23°C.**

Additive	quantity added (mg/IU heparinase)	$t_{1/2}$ (h)
water	ND	4.00
PBS (10 mM phosphate, 150 mM NaCl)	ND	8.40
dextran	5.0	11.52
$\beta$ -cyclodextrin	5.0	13.42
sucrose	5.0	9.12
glucose	5.0	12.48
galactose	5.0	15.05
galactosamine	5.0	36.00
polyethylene glycol	5.0	8.54
albumin	0.5	18.19
cysteine	0.1	38.40
ammonium sulfate	13.2	486.96
calcium chloride	0.6	196.80

Subsequently, the effect of varying quantities of lyophilized ammonium sulfate on coagulation was examined. Quantities of ammonium sulfate up to 2.5 mg/ml blood had no measurable effect on coagulation. As little as 0.2 mg ammonium sulfate per 1.5 IU heparinase is able to extend the half life of lyophilized enzyme to at least 200 days at 23°C. In this embodiment, 0.5 to 2.0 IU heparinase is lyophilized in the presence of 1.0 mg ammonium sulfate into tubes capable of containing 0.2 to 5.0 ml liquid. These may include a variety of tubes ranging from microtitre wells to 5.0 ml polypropylene test tubes. In other embodiments the heparinase-ammonium sulfate can be lyophilized directly into containers included in various commercially available hematological assay kits, into syringes which are used to collect the sample to be tested, into transfer pipets or into sterile vials with septum closures in which the preparation could be resolubilized prior to use as an injectable therapeutic agent.

**Example 3: Neutralization of heparin in samples for ACT assays.**

*ACT assays.* The activated clotting time (ACT) assay is routinely employed in cardiovascular surgery to monitor the anticoagulant effects of heparin. As a functional measure of the intrinsic clotting pathway, ACT measurements are influenced by factors other than heparin.

Cardiovascular procedures and extracorporeal circulation may lead to incidents of platelet dysfunction, hemodilution, disseminated intravascular coagulation, or hypercoagulation which alter the baseline clotting time during the course of an operation. Therefore, assuming a constant baseline ACT equivalent to the pre-operative value may lead to inaccurate conclusions concerning the administration of heparin or protamine. For example, hemodilution results in an extended ACT and may lead to an overestimate of the quantity of protamine required for neutralization. Conversely, hypercoagulation, induced by contact activation, results in a shortened ACT and may lead to an underestimate of the quantity of protamine required for neutralization.

The expeditious pre-treatment of samples to remove heparin prior to the determination of the ACT would allow for the procurement of baseline clotting information, even on sample taken from patients who have received heparin. 1.5 IU heparinase was lyophilized as described above into one channel of the dual test high range ACT cartridge manufactured by HemoTec, Inc. (Denver, CO). The resulting cartridge yields two test results: the activated clotting time of an untreated sample and the activated clotting time of a sample treated with heparinase. Freshly drawn blood was either directly assayed with these cartridges or incubated with 6 IU heparin/ml prior to the test. The results shown in Table 4 indicate that heparinase completely neutralized the heparin contained in the 6 IU/ml blood sample. The sample treated with both heparin and heparinase gave an identical result to a sample that had not

been exposed to either reagent. Similar results are obtained over the range of 0-12 IU heparin/ml. The upper limit heparin dose observed in cardiovascular surgery is 6 IU/ml.

**Table 4: Neutralization of heparin in whole human blood.**

sample	heparin (IU/ml)	heparinase (IU/ml)	ACT (sec)
whole blood	0	0	$131.3 \pm 1.3$
whole blood	0	1.5	$128.0 \pm 4.7$
whole blood	6	0	> 999
whole blood	6	1.5	$131.3 \pm 4.3$

This assay will provide the clinician with valuable information during cardiovascular surgery including answers to the following:

- 1) Is heparin present pre-surgically?
- 2) Has the baseline clotting time varied during the procedure?
- 3) Has protamine reversal of heparin been achieved?
- 4) Has heparin rebound occurred post-surgically?

The results from a clinical study and interpretations of potential test results are shown in Table 5.

**Table 5: Uses of the heparinase activated clotting time test in cardiovascular surgery.**

<b>Time of Test</b>	<b>ACT result</b>	<b>Heparinase ACT result</b>	<b>Condition (19 cases)</b>	<b>Recommended Action</b>	<b>study results</b>
<b>Pre-bypass</b>					
	normal	normal	normal coagulation	none	16/19
	above normal	normal	heparin present	none	0/19
	above normal	normal	coagulopathy	further evaluation	3/19
<b>On bypass</b>					
	≥ 450	normal	anti-coagulated	none	19/19
	≥ 450	above normal	hemodilution	hemoconcentrate add coagulation factors and heparin	0/19
	below 450	normal	insufficient anti-coagulant	add heparin	0/19
<b>Post-protamine</b>					
	normal	normal	adequate reversal	none	4/19
	above normal	above normal	hemodilution or coagulopathy	further evaluation add coagulation factors	0/19
	above normal	normal	heparin present	add protamine	0/19
	below normal	below normal	hyper-coagulation	anticoagulant therapy	15/19
	below normal	normal	hypercoagulation and heparin present	none	0/19
<b>ICU</b>					
	normal	normal	normal	none	ND
	above normal	normal	heparin rebound	add protamine	ND
	above normal	above normal	coagulopathy	further evaluation	ND

Normal = 105 - 130 seconds

**Example 5:        Neutralization of heparin in clinical samples for PT and aPTT assays.**

*PT/aPTT.* Prothrombin time (PT) and activated partial thromboplastin time (aPTT) assays are routinely employed in the assessment of hemostatic function. PT is performed by adding a preparation of phospholipid, tissue factor and calcium to the patient's citrated plasma and determining the time required for a clot to form. This test measures the aggregate activity of factors VII, X, V and fibrinogen. The PT is often used to follow oral anticoagulant therapies, i.e., coumarin and anti-vitamin K drugs. The aPTT test is performed by adding a preparation of celite, phospholipid, and calcium to the patient's citrated plasma and determining the time required for a clot to form. This test measures the aggregate activity of factors XII, XI, IX, VIII, X, II, V and fibrinogen. The aPTT is commonly used as a coagulation screening test for factor dysfunction in hospitalized patients.

A method for determining heparin independent aPTT and PT measurements would aid in the evaluation of these results. In an analogous fashion to the development of the heparinase ACT test, the use of heparinase to provide baseline aPTT/PT information has been investigated. 1.5 IU heparinase was lyophilized into 1.5 ml polypropylene tubes as described above. This quantity of heparinase was able to completely reverse the effects of up to 1.0 IU/mL heparin in citrated human plasma, as shown in Table 6. The typical therapeutic heparin dose is 0.3 IU/mL.

Table 6: sample	Neutralization of heparin in human plasma.			
	heparin (IU/ml)	heparinase (U/ml)	aPTT (sec)	PT (sec)
human plasma	0	0	25.7 $\pm$ 0.1	12.4 $\pm$ 0.3
human plasma	0	100	24.3 $\pm$ 0.1	12.2 $\pm$ 0.1
human plasma	0.5	0	87.6 $\pm$ 0.1	13.9 $\pm$ 0.3
human plasma	1.0	100	25.9 $\pm$ 0.2	12.7 $\pm$ 0.2
human plasma	1.0	0	299.0 $\pm$ 22	14.8 $\pm$ 0.1
human plasma	1.0	100	26.6 $\pm$ 0.2	12.4 $\pm$ 0.1

Whenever baseline coagulation data is desired, a 0.5 to 1.0 mL plasma aliquot, can be added to the vial and gently mixed by inversion prior to standard coagulation analysis.

**Example 7: *In vivo* Neutralization of heparin.**

The previous unsuccessful attempts to neutralize heparin *in vivo*, reported by Klein, et al., (1982) and Langer, et al., (1982), appears to reflect the same phenomenon described above with respect to the *F. heparinum* anticoagulant moiety. Using heparinase purified by the method described herein, the *in vivo* investigation was repeated and complete heparin reversal was achieved within 15 minutes. The results are shown in Figure 1.

A 4 kg rabbit was administered 250 IU/kg heparin extending the activated clotting time (ACT) from 180 to greater than 999 seconds. Within 5 minutes of heparin administration, 2.5 IU/kg heparinase was injected and the ACT monitored periodically over the following hour. Fifteen minutes after the heparinase injection, the ACT returned to baseline indicating a complete reversal of heparin's anticoagulant activity. The control rabbit, receiving the same amount of heparin, but no heparinase, returned to baseline ACT only after 1.5 hours.

This data indicates the potential for using direct heparinase injections as a method to reverse heparin in cardiovascular surgery.

We claim:

1. A method to eliminate the physiological effects of heparin on blood components comprising treating blood containing heparin with a stabilized heparinase preparation, free of an anticoagulant component, having optimal activity at  $\text{pH} = 6.5$  to  $7.0$ ;  $[\text{NaCl}] = 0.1$ , and  $37^\circ\text{C}$ .
2. The method of Claim 1 wherein the heparinase is purified from cultures of *F. heparinum* by affinity chromatography using a polysulfated resin.
3. The method of Claim 2 wherein the partially purified heparinase is applied to the affinity resin under conditions in which the heparinase is retained by the resin and a *F. heparinum* component inhibiting coagulation is not retained by the resin.
4. The method of Claim 2 wherein the heparinase is eluted from the affinity resin by increasing the conductivity of the eluent such that the eluted heparinase fraction is free of a *F. heparinum* component inhibiting coagulation.
5. The method of Claim 1 wherein the blood contains low molecular weight heparins.
6. The method of Claim 1 wherein the heparinase formulation is prepared by lyophilizing  $0.05$  to  $3.0$  IU anticoagulant free heparinase in the presence of  $0.5$  -  $1.0$  mg ammonium sulfate/IU heparinase into containers suitable for the collection or incubation of blood or plasma samples.
7. The method of Claim 6 wherein the containers are selected from the group consisting of blood collection tubes, pipet tips, fluid transfer devices and syringes.

8. The method of Claim 1 wherein the blood or plasma sample is incubated with the lyophilized heparinase formulation for between 1 and 3600 seconds at a temperature between 2 and 41 °C prior to the initiation of a hematological test.

9. The method of Claim 8 wherein the test relies on the formation of clot as its endpoint.

10. The method of Claim 8 wherein the test relies on the action of a blood component whose action is influenced in the presence of heparin in such a manner as to alter the test result.

11. The method of Claim 8 wherein the test relies on the action of a blood component whose action is influenced in the presence of low molecular weight heparins in such a manner as to alter the test result.

12. The method of Claim 1 wherein the heparinase formulation is prepared by lyophilizing 0.05 to 300 IU anticoagulant free heparinase in the presence of 0.5 - 1.0 mg ammonium sulfate/TU heparinase into a container further comprising resolubilizing the heparinase with a physiologically compatible solution.

13. The method of Claim 1 further comprising the dissolved heparinase formulation is injected intravenously into a patient receiving heparin therapy for the purpose of neutralizing the anticoagulant properties of heparin in the bloodstream.

14. The method of Claim 13 wherein the dissolved heparinase formulation is injected intravenously into a patient receiving low molecular weight heparin therapy for the purpose of neutralizing the anticoagulant properties of low molecular weight heparin in the bloodstream.

15. A stabilized heparinase preparation, free of an anticoagulant component, having optimal activity at pH = 6.5 to 7.0; [NaCl] = 0.1, and 37°C.

16. The heparinase preparation of claim 15 derived from *F. heparinum*.

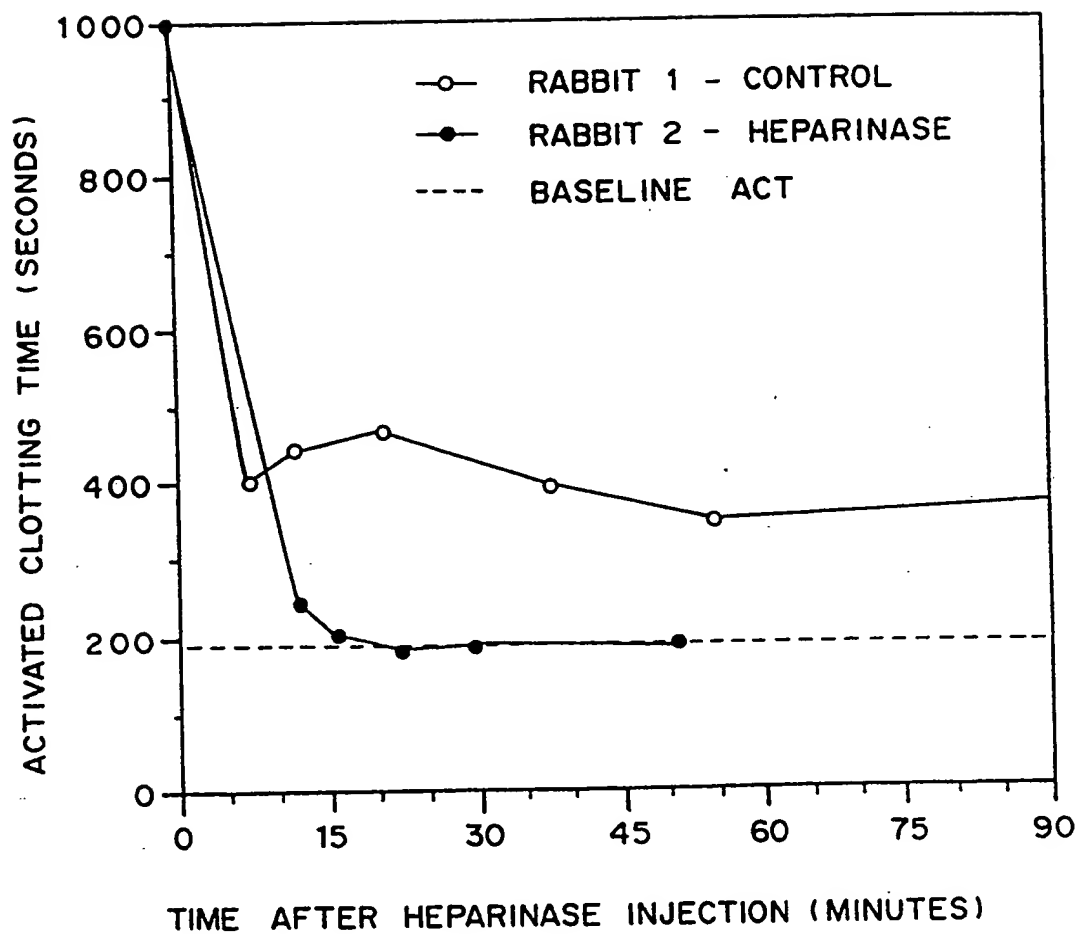
17. The heparinase preparation of claim 15 wherein the heparinase is purified from cultures of *F. heparinum* by affinity chromatography using a polysulfated resin.

18. The heparinase preparation of Claim 15 wherein the partially purified heparinase is applied to the affinity resin under conditions in which the heparinase is retained by the resin and a *F. heparinum* component inhibiting coagulation is not retained by the resin.

19. The heparinase preparation of Claim 18 wherein the heparinase is eluted from the affinity resin by increasing the conductivity of the eluent such that the eluted heparinase fraction is free of a *F. heparinum* component inhibiting coagulation.

20. The heparinase preparation of Claim 15 wherein the heparinase formulation is prepared by lyophilizing 0.5 - 1.0 mg ammonium sulfate/IU heparinase.

21. The heparinase preparation of Claim 20 wherein 0.05 to 3.0 IU anticoagulant free heparinase is lyophilized in the presence of 0.5 - 1.0 mg ammonium sulfate/IU heparinase into containers suitable for the collection or incubation of blood or plasma samples.

*FIGURE 1*

**I. CLASSIFICATION OF SUBJECT MATTER** (If several classification symbols apply, indicate all)<sup>4</sup>

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5

A 61 K 37/56

C 12 N 9/88

C 12 Q 1/56

G 01 N 33/86

**II. FIELDS SEARCHED**Minimum Documentation Searched<sup>7</sup>

Classification System

Classification Symbols

Int.Cl.5

C 12 N

A 61 K

C 12 Q

Documentation Searched other than Minimum Documentation  
to the Extent that such Documents are Included in the Fields Searched<sup>8</sup>**III. DOCUMENTS CONSIDERED TO BE RELEVANT<sup>9</sup>**

Category <sup>10</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>1</sup>
A	J. Lab. Clin. Med., vol. 79, no. 6, June 1972, E.D. HUTT et al.: "Use of heparinase to eliminate heparin inhibition in routine coagulation assays", pages 1027-1035, see page 1027, paragraph 1 - page 1028, paragraph 1; page 1028, paragraph 3; page 1029, paragraph 2 - page 1033, paragraph 1 (cited in the application) ---	1,2,9, 10,13, 15,16
A	Thrombosis Research, vol. 60, no. 4, 15 November 1990, L.H. BÜHMER et al.: "Heparin degradation by a novel microbial heparinase", pages 331-336, see page 331, paragraph 2 - page 332, paragraph 1; page 332, paragraph 2 - page 333, paragraph 1; page 334, paragraphs 1-2 (cited in the application) -----	1,9,10, 13,15
	-----	-/-

<sup>10</sup> Special categories of cited documents:<sup>10</sup> "A" document defining the general state of the art which is not  
considered to be of particular relevance<sup>10</sup> "E" earlier document but published on or after the international  
filing date<sup>10</sup> "L" document which may throw doubts on priority claim(s) or  
which is cited to establish the publication date of another  
citation or other special reason (as specified)<sup>10</sup> "O" document referring to an oral disclosure, use, exhibition or  
other means<sup>10</sup> "P" document published prior to the international filing date but  
later than the priority date claimed<sup>10</sup> "T" later document published after the international filing date  
or priority date and not in conflict with the application but  
cited to understand the principle or theory underlying the  
invention<sup>10</sup> "X" document of particular relevance; the claimed invention  
cannot be considered novel or cannot be considered to  
involve an inventive step<sup>10</sup> "Y" document of particular relevance; the claimed invention  
cannot be considered to involve an inventive step when the  
document is combined with one or more other such docu-  
ments, such combination being obvious to a person skilled  
in the art.<sup>10</sup> "Z" document member of the same patent family**IV. CERTIFICATION**

Date of the Actual Completion of the International Search

07-08-1992

Date of Mailing of this International Search Report

12.10.92

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

MONTERO LOPEZ B.

## III. DOCUMENTS CONSIDERED TO BE RELEVANT

(CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	WO,A,8705333 (THE CHILDREN'S MEDICAL CENTER CORP.) 11 September 1987, see page 3, line 31 - page 4, line 17; page 5, line 22 - page 6, line 13; page 6, line 22 - page 7, line 17; page 9, lines 6-20, & US,A,4795703 (cited in the application) ---	1,2,6-8,15,16,20,21
A	WO,A,8802400 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 7 Apr 11 1988, see page 2, lines 9-16,22-31; page 3, line 20 - page 4, line 10; page 5, lines 14,20; table I; page 9, line 25 - page 10, line 24; page 11, line 5 - page 14, line 19 ---	1,2,5,9-11,15,16
A	EP,A,0370958 (RESEARCH DEVELOPMENT CORP. OF JAPAN) 30 May 1990, see page 2, lines 35-39; page 7, lines 14-42; example 5 -----	1,2

**Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **13,14**  
because they relate to subject matter not required to be searched by this Authority, namely:  
**Although Claims 13,14 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition**
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9202724  
SA 59872

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 01/09/92. The European Patent Office is in no way liable for those particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO-A- 8705333	11-09-87	US-A- 4795703	03-01-89
		DE-A- 3778616	04-06-92
		EP-A, B 0259463	16-03-88
		JP-T- 63503194	24-11-88
WO-A- 8802400	07-04-88	AU-A- 8102987	21-04-88
EP-A- 0370958	30-05-90	JP-A- 2142470	31-05-90

IPO FORM 7079

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82

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